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Biomimetic graphene oxide-cationic multi-shaped gold nanoparticle-hemin hybrid nanozyme: Tuning enhanced catalytic activity for the rapid colorimetric apta-biosensing of amphetamine-type stimulants

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Abstract

Amphetamine-type stimulants are a class of illicit drug that constitutes a worldwide problem to which intelligence agencies, first responders and law enforcement are tasked with identifying them in unknown samples. We report on the development of a graphene oxide (GO)-cationic multi-shaped gold nanoparticle (AuNP)-hemin hybrid nanozyme as a new biomimetic catalytic-induced aptamer-based colorimetric biosensor platform for amphetamine (AMP) and methamphetamine (MAMP). GO was electrostatically bonded to cationic multi-shaped cetyltrimethylammonium bromide (CTAB)-AuNPs to form a GO-CTAB-AuNP hybrid nanozyme exhibiting enhanced catalytic activity in the presence of hemin. The binding of an MNS 4.1 anticocaine DNA aptamer on the GO-CTAB-AuNP-hemin nanozyme assembly and the subsequent catalytic oxidation by 3,3,5,5-tetramethylbenzidine in the presence of H₂O₂ ensured that the colorimetric reaction was tuned to selectively detect AMP and MAMP with high sensitivity. Under optimum experimental conditions, AMP and MAMP were quantitatively detected within 1 min with a detection limit of 34.1 ng/mL and 28.6 ng/mL respectively. Selected substances and drugs, known to react positively

to Marquis and Mandelin reagents (used in AMP and MAMP presumptive testing) and well-known adulterants, were tested for their affinity to react with the aptamer-based GO-CTAB-AuNP-hemin peroxidase mimic biosensor. The deep blue colorimetric reaction, specific to AMP and MAMP detection, was used as the basis to affirm the selectivity of the aptamer-based GO-CTAB-AuNP-hemin peroxidase mimic biosensor. We believe the colorimetric biosensor developed in this work demonstrates a promising new direction in presumptive testing for AMP and MAMP.

Keywords: Aptamer; Gold nanoparticle; Graphene oxide; Hemin; Peroxidase mimic

1 Introduction

The outcome of investigative cases relating to the illegal possession of illicit drugs is heavily dependent on test results which provide scientific evidence that the suspected person is in possession of the drug in question. Colour spot tests, more commonly referred to as presumptive tests, are useful in providing rapid qualitative identification that a controlled drug may be present in a sample, enabling prompt action at the point of seizure [1–3]. The production, consumption and commercialization of illicit substances remain a serious concern in most countries. Within the context of production and exploitation, amphetamine-type stimulants (ATS) such as methamphetamine (MAMP) and amphetamine (AMP) dominate the illicit drug market in many countries [4] where the amount of confiscated ATS has drastically increased in recent years [5–7].

Colorimetric spot test such as the Marquis, Simon's and Mandelin tests are the 'gold' standard test used in ATS presumptive analysis. However, despite their wide-spread use in forensic analysis, there have been several reports of false positive results generated from chemical analysis of everyday items such as Epsom salts [8] and Krispy Kreme crumbs [9]. The limitation of colour spot test was recently brought to light in a case where unknown heart-shaped drugs were seized in Italy and presumptively identified as ATS using the Marquis reagent. However, further confirmatory test unraveled the substances as containing methyltestosterone, methanedone and androgen steroids with no trace of ATS being present [10,11]. Poor sensitivity of the colorimetric test when there are low levels of target analogue may be a significant contributing factor to false negative results [12]. In addition, the handling of colour spot reagents also poses increased health risk as many of the chemicals being used are highly corrosive and toxic. There is therefore an increased interest in the development of highly selective, ultrasensitive, rapid and safe-to-use colour spot test for accurate on-site and point of seizure ATS detection and one avenue of exploration to address this lies in the use of nanotechnology-based biosensors.

Over the last decade, nanomaterials-based artificial enzymes, known as nanozymes have emerged as a powerful alternative to natural enzymes in various applications ranging from pollutant removal, stem cell growth, cancer diagnostics and biosensing [13–15]. In the field of biosensing particularly, nanozyme-based peroxidase mimics involving the use of carbon-based nanomaterials [16–19], magnetic Fe₃O₄ nanoparticles (MNPs) [19] and plasmonic nanoparticles NPs [20] as catalyst to catalyse the oxidation of a suitable substrate in the presence of hydrogen peroxide (H₂O₂) have been exploited in several colorimetric assays [21–23]. Gold NPs (AuNPs), characterized by their surface plasmon resonance (SPR) absorption feature are known to change

colour relative to their chemical state and this property has been exploited in colorimetric assays [24]. Graphene oxide (GO) on the other hand, is a water-soluble 2D carbon nanomaterial, characterized by sheets of arranged carbon atoms in a honeycomb-like lattice structure and characterized by oxygen functional moieties (e.g. epoxy, carbonyl, carboxyl and hydroxyl) [25]. Despite the peroxidase mimic activity of AuNPs and graphene, their catalytic efficiency in sensor technology can be enhanced further by forming hybrid nanostructures [26,27]. Additionally, the incorporation of hemin (iron protoporphyrin IX) in a peroxidase mimic assay can also aid catalytic enhancement of the sensor. Hemin, known as the active site in heme-containing protein such as peroxidase, myoglobin, hemoglobin and catalases [28], exhibits peroxidase-like catalytic activity through the oxidation of a suitable substrate by H_2O_2 [29].

To selectively target the analyte of interest, the incorporation of a receptor molecule within a nanozyme peroxidase mimic assay is an efficient way to achieve this. Aptamers are short single-stranded RNA or DNA sequences, capable of undergoing selective antigen interaction due to three-dimensional structure formation [30]. Intermolecular interaction with the target of interest via aromatic ring stacking, hydrogen bonding or van der Waal forces is facilitated by the nucleic acid aptamer structure (which could be a loop, quadruplex motifs, pseudoknot or k-turn) [31]. Compared with traditional antibodies, aptamers exhibit greater thermal stability, longer shelf-lives with no loss of activity and can easily be transported and stored [32].

In this work, we report on a novel catalytic-enhanced peroxidase mimic biosensor for the colorimetric detection of AMP and MAMP using GO-multi-shaped cetyltrimethylammonium bromide (CTAB)-AuNPs hybrid nanozyme with DNA aptamer as a bioreceptor and hemin as a catalytic signal amplifier. GO was first bound to CTAB-AuNPs via electrostatic interaction to form a GO-CTAB-AuNP hybrid nanozyme complex. Then a DNA aptamer, specific to the target ATS was thereafter bound to the GO-CTAB-AuNP hybrid nanozyme surface to capture the target ATS. This was followed by the binding of hemin and the subsequent catalytic oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) by H_2O_2 to generate a deep blue colour specific to the target ATS. To the best of our knowledge, the aptamer-based GO-CTAB-AuNP-hemin peroxidase mimic biosensor for ATS is the first of its kind and can colorimetrically detect AMP and MAMP as individual compounds in ~1 minute (min) with high sensitivity and selectivity.

2 Experimental

2.1 Materials

± Methadone HCl ($\geq 98\%$), acetylsalicylic acid, acetaminophen, silver nitrate ($AgNO_3$), (+) MAMP hydrochloride, d-AMP, BIS-TRIS, Trizma® acetate and H_2O_2 (30% w/w) in solution with stabilizer were purchased from Sigma Aldrich. Tris(hydroxymethyl)aminomethane was purchased from Formedium. Dimethyl sulfoxide (DMSO), ascorbic acid, TMB, MES, CTAB, potassium acetate (KAc) and gold (III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$), graphite powder and potassium permanganate ($KMnO_4$) were purchased from Thermo Fisher. 3,6-Diacetylmorphine and codeine HCl were purchased from lipomed. The thiolated DNA aptamer oligonucleotide: The AMP and MAMP-specific DNA aptamer with the nucleic acid sequence;

HSC₆-5'-ACGGTTGCAAGTGGGACTCTGGTAGGCTGGGTAATTTGG [33] was synthesized and purified by Eurofins. All other chemicals were used as received. The buffer solution used in this study was prepared in

Milli-Q water.

2.2 Characterization

UV/vis absorption measurements were carried out on a Cary Eclipse (Varian) spectrophotometer. Transmission electron microscopy (TEM) measurements were performed on a JEOL JEM-1200EX operated at 80 kV. Scanning electron microscope (SEM) imaging was carried out using a JEOL JSM 7400F field emission SEM. Zeta potential measurements were carried out using a Brook haven Nanobrook Omni particle size and zeta potential analyser. Absorbance measurements were recorded on an 800 TS microplate absorbance reader from BioTek. The Raman spectra were collected using an in-house built microprobe system equipped with a continuous wave laser sources emitting at 633 nm, the Oriel MS257 monochromator fitted with the Andor Newton EMCCD detector TE cooled to -70°C . The backscattering configuration was used for the signal collection. The incident power on the samples was 7 mW. The spectra were recorded using a 40 \times objective, a 1 s accumulation time with a total of 10 accumulations.

2.3 Synthesis of multi-shape CTAB-AuNPs

Multi-shape CTAB-AuNPs were synthesized via the seed-mediated approach with modifications [34]. The seed solution was prepared by mixing 10 mL 0.1 M CTAB, 5 mL 2.5×10^{-4} M $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ and 0.6 mL 0.01 M NaBH_4 . Thereafter, 150 μL of the seed solution was added into the growth solution containing a mixture of 10 mL 0.1 M CTAB, 0.4 mL 0.1 M ascorbic acid, 0.5 mL 0.004 M AgNO_3 and 5 mL 2.5×10^{-4} M $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$. The solution was stirred for few min and kept in the dark for ~ 24 hours. Purification of the NPs was carried out via centrifugation.

2.4 Synthesis of GO nanosheets

GO nanosheets were synthesized via the modified Hummers method [35,36]. Graphite oxide was firstly prepared by mixing 2.5 g graphite powder and 1.25 g NaNO_3 in ice-cooled solution of 60 mL H_2SO_4 . Once the graphite powder was thoroughly dispersed in the acidified solution, 7.5 g KMnO_4 was added and the solution was kept in ice for ~ 2 h and subsequently stirred for ~ 24 h at room temperature. The reaction mixture was then placed in an ice bath and 75 mL of Milli-Q water was added slowly. The reaction mixture was thereafter stirred for ~ 24 h at $\sim 100^{\circ}\text{C}$ and allowed to cool down prior to the slow addition of 25 mL 35% H_2O_2 . Graphite oxide was thereafter purified by centrifugation with 5% HCl followed by acetone and dried in the fume hood. Graphite oxide was exfoliated to GO via ultrasonication.

2.5 Bioassay procedure

NaAc-KAc-KCl-HCl is a novel buffer solution developed in this work and was used as the choice buffer for the ATS catalytic assay. It was prepared by mixing 0.25 g NaAc, 0.25 g KAc, and 0.37 g KCl in 50 mL of MilliQ water. Then, 6.7 mL 0.1 M HCl was added into the 50 mL buffer solution and made up to 100 mL with MilliQ water and the pH of the solution was adjusted to 2.2. Electrostatic interaction between GO nanosheets and CTAB-AuNPs was formed by mixing 0.2 mg/mL GO with 0.04 nM CTAB-AuNPs. The final concentration of the GO-CTAB-AuNP hybrid was 0.03 nM. Thereafter, 40 μL of GO-CTAB-AuNP hybrid solution was mixed with 5 μL DNA aptamer (100 μM), 150 μL of target ATS concentration in KCl-HCl-

NaAc-KAc buffer, pH 2.2, 5 μL hemin (100 μM), 90 μL TMB (3000 μM) and 60 μL H_2O_2 (1.2 M) in a 96-well plate. The absorbance of the solution was recorded at ~ 1 min after adding H_2O_2 using a BioTek 800 TS microplate reader with a 630 nm filter.

3 Results and discussion

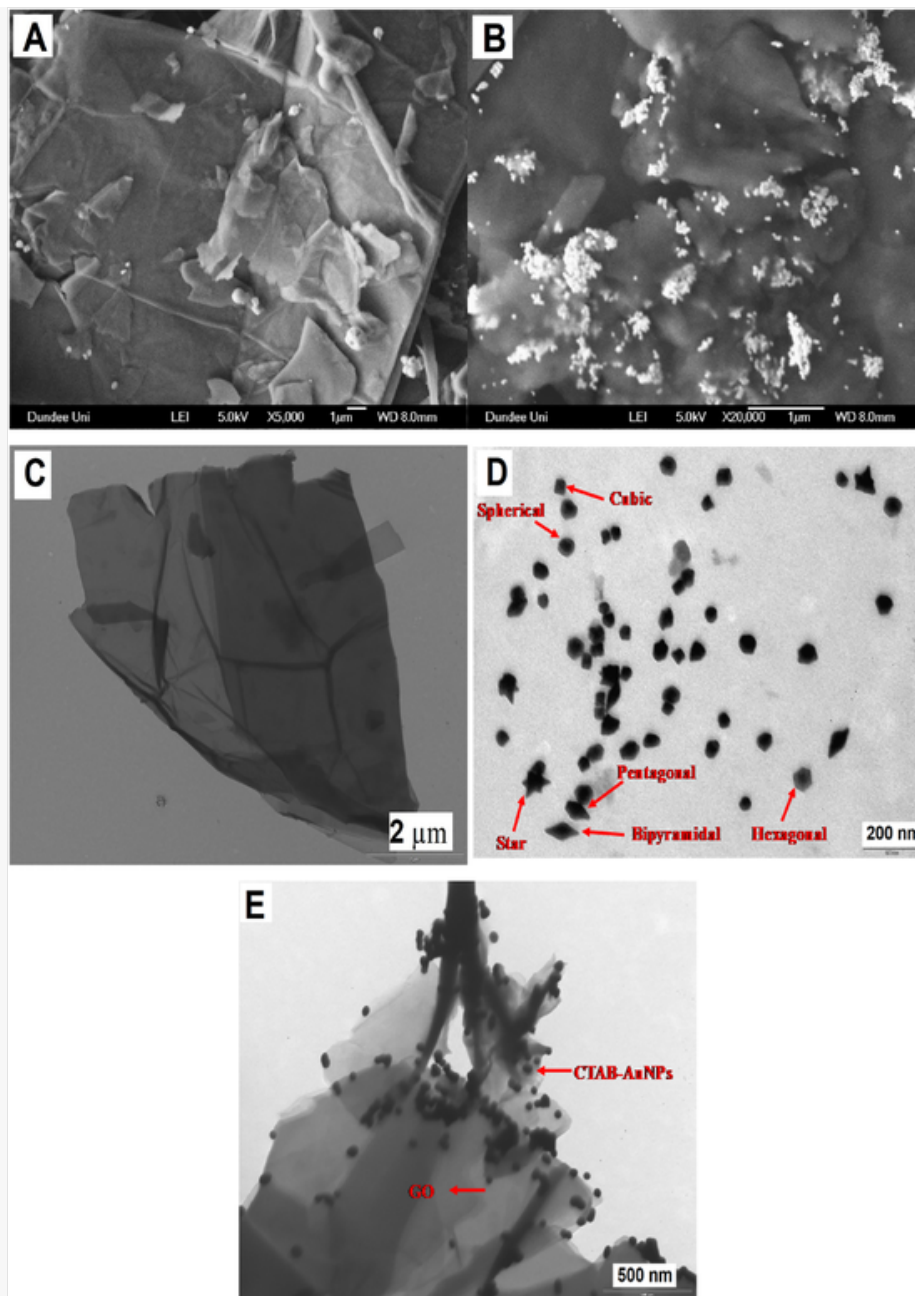
3.1 Structural properties

3.1.1 SEM analysis

Graphene, known to be conceptually flat, is characterized by an sp^2 trigonal-bonded hybridized array of carbon atom sheets. It is typically synthesized by chemical exfoliation of graphite oxide under strong oxidative and acidic reaction conditions and is characterized by oxygen atoms that are embedded in functional groups such as carboxyls, hydroxyls, epoxides and esters which lie both on the graphene sheet edges and on the basal plane [37,38]. GO is stabilized as a colloidal solution in water and is rendered negatively charged when the groups at the sheet edges, particularly carboxyl groups are ionized [37]. Fig. 1A and B shows the SEM images of GO and GO-CTAB-AuNP nanohybrid. From Fig. 1A, GO is characterized by crumpled thin and randomly aggregated flaky sheets, stacked together with observed folding and wrinkles. The corresponding GO-CTAB-AuNP nanohybrid SEM image shown in Fig. 1B, revealed the retained morphology of GO and the embedded CTAB-AuNPs across the surface sheet.

alt-text: Fig. 1

Fig. 1



SEM images of (A) GO and (B) GO-AuNP nanohybrid. TEM images of (C) GO, (D) CTAB-AuNPs and (E) GO-AuNP nanohybrid.

3.1.2 TEM analysis

TEM analysis was used to further probe the surface morphology of GO, GO-CTAB-AuNP nanohybrid and the positively charged CTAB-AuNPs. Fig. 1C shows that the TEM micrograph of GO is characterized by well-exfoliated individual sheets without the presence of bulk aggregates. The TEM image of the multi-shaped CTAB-AuNPs (Fig. 1D) synthesized via the seed-mediated approach, reveals a well-dispersed size distribution and homogeneity with a mixture of cubic, spherical, star, bipyramidal, hexagonal and irregular-shaped particles. The formation of various shaped particles is understood to arise from the interplay between the growth kinetics and the faceting properties of the CTAB surfactant [34]. The average particle size of CTAB-

AuNPs was 47 nm. The corresponding TEM image of GO-CTAB-AuNP is shown in Fig. 1E. From the TEM micrograph, anchored CTAB-AuNPs were seen embedded within the sheet layer of GO, thus revealing the strong binding interaction between GO and CTAB-AuNPs.

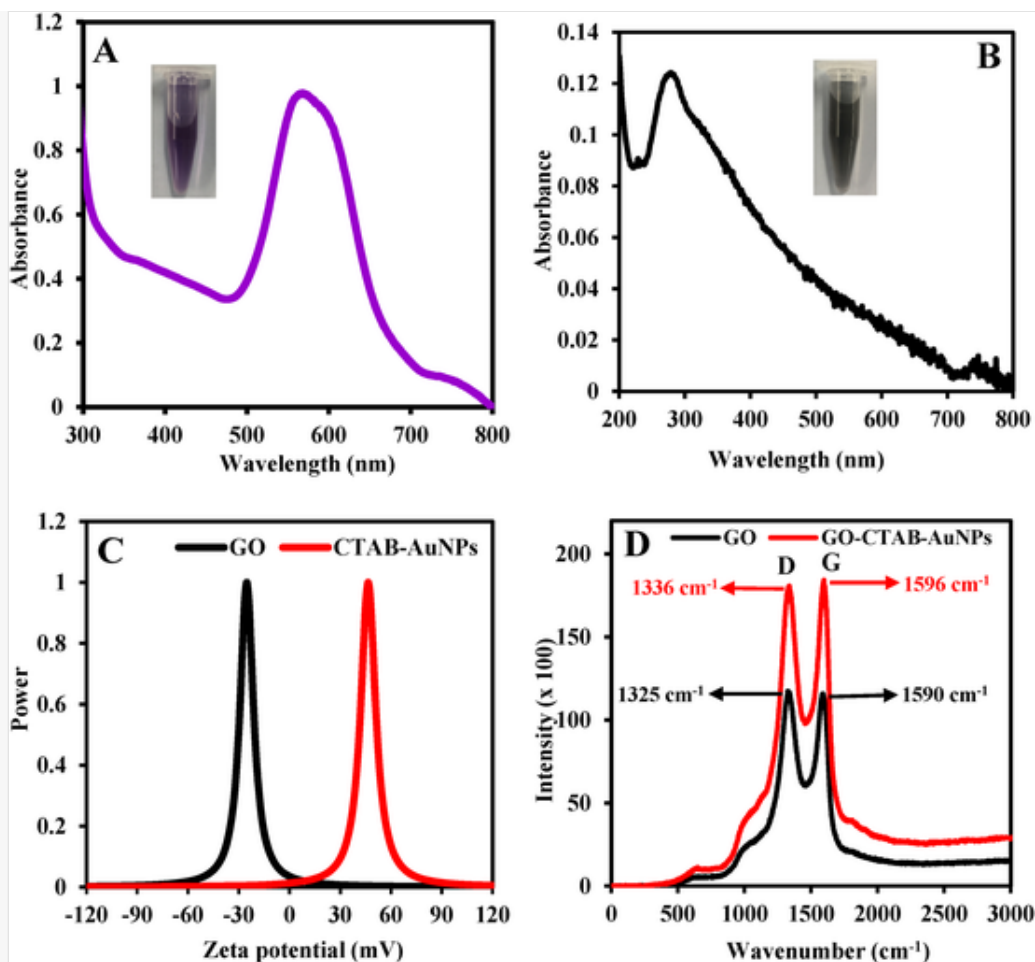
3.2 Optical properties

3.2.1 UV/vis absorption

The optical properties of AuNPs are known to be determined by the presence of SPR absorption band. Any change in the geometry, shape or size of the particles influences the local electron confinement, resulting in alterations of the SPR band position and consequently the colour change of the colloidal solution [39,40]. Fig. 2A shows that the multi-shaped CTAB-AuNPs solution is characterized by a distinctive SPR absorption band peaked at ~570 nm. Due to the altered shape of the NPs, the SPR band of the synthesized colloidal solution appears to be red-shifted compared with that of Au nanospheres (peaked at a wavelength of ~520 nm) and blue-shifted to that of anisotropic Au nanorods (>600 nm) [41]. The inset of Fig. 2A shows the strong purple colloidal solution of the synthesized CTAB-AuNPs. For GO (Fig. 2B), the characteristic sharp absorption peak at ~279 nm is assigned to the $\pi \rightarrow \pi^*$ transition of the sp^2 C=C bond [42] while the broad peak shoulder at ~310–380 nm is assigned to the $n \rightarrow \pi^*$ transition of the C-O-C (epoxide) and R-O-O-R (peroxide) functional moieties [43].

alt-text: Fig. 2

Fig. 2



UV/vis absorption spectra of (A) multi-shaped CTAB-AuNPs and (B) GO nanosheet. ZP curve (C) and Raman spectra (D) of GO and CTAB-AuNPs. Inset of Fig. 2B = picture of CTAB-AuNPs in solution and inset of Fig. 2C = picture of GO in solution.

3.2.2 Zeta potential

The amount of work needed to move a unit positive charge (without acceleration) with affinity to the NP surface is reflected in the electric field potential. The electric field potential of a colloidal NP solution at the slipping/shear plane of an applied electric field is known as the Zeta potential. The adsorbed double layer, known as the electric double layer (EDL) is created on the NP surface when the colloidal NP solution is dispersed in solution. The EDL inner layer is embedded with ions/molecules having opposite charge to the colloidal NP solution. Therefore, the principle of Zeta potential relates to the difference in the electric field potential between the surrounding layer of dispersant and the EDL of an electrophoretic mobile particle [44]. The Zeta potential of GO and CTAB-AuNPs were measured to confirm the surface charge of the respective nanomaterials. From Fig. 2C, the Zeta potential curves reveal the negative charge potential of GO with a Zeta potential charge of -27.61 mV and the positive charge potential of CTAB-AuNPs with a Zeta potential charge of $+46.49$ mV.

3.2.3 Raman analysis

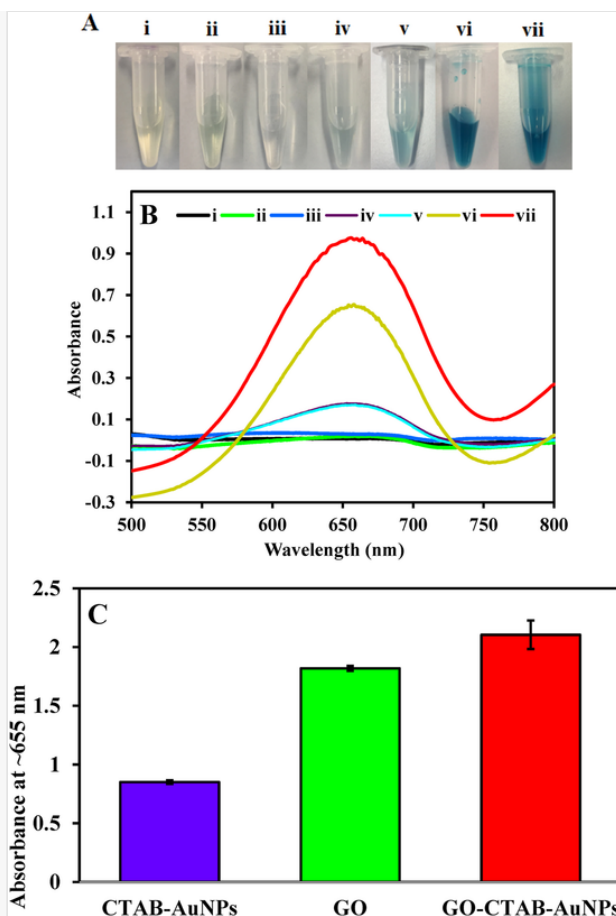
The Raman spectra of GO and GO-CTAB-AuNP shown in Fig. 2D are characterized by two distinct bands known as the D and G band [45]. For GO, the peak at 1325 cm^{-1} is ascribed to the D band and it arises due to the reduction in size of the sp^2 domain plain induced from strong oxidation of graphene nanosheets. The second peak at 1590 cm^{-1} is ascribed to the G band and it arises from the $\text{E}_{2\text{g}}$ first-order scattering [45]. An increase in intensity and a slight shift of the D (1336 cm^{-1}) and G band (1596 cm^{-1}) to higher wavenumbers was observed for the GO-CTAB-AuNP nanohybrid relative to GO. The observed change can be attributed to the electrostatic binding interaction between GO and CTAB-AuNPs. Prior to the electrostatic binding interaction, the intensity ratio ($I_{\text{D}}/I_{\text{G}}$) for GO was 1.01 but decreased slightly to 0.98 for the GO-CTAB-AuNP nanohybrid.

3.3 Catalytic activity

The peroxidase mimic activity of the aptamer-based GO-CTAB-AuNP-hemin biosensor in inducing a catalytic colour reaction for the target ATS was investigated. From the UV/vis absorption data (Fig. 3A and B), no catalytic response and visible colour change was observed for TMB/ H_2O_2 , GO-CTAB-AuNP + TMB/ H_2O_2 and GO-CTAB-AuNP + AMP. However, we observed a weak catalytic response when the GO-CTAB-AuNP hybrid nanozyme was reacted with the DNA aptamer, target ATS and TMB/ H_2O_2 but without the catalytic amplifying effect of hemin in the biosensor system. With hemin incorporated into the catalytic biosensor system, a strong catalytic signal and blue colorimetric reaction, characterized by a distinct absorption peak at $\sim 660\text{ nm}$, was observed for both AMP and MAMP. It is evident that the strong colorimetric and corresponding catalytic response can be attributed to the signal-enhancing effect of hemin in the biosensor system.

alt-text: Fig. 3

Fig. 3



(A) Photographic colorimetric picture and (B) corresponding UV/vis absorption spectra of (i) TMB/H₂O₂, (ii) GO-AuNPs + TMB/H₂O₂, (iii) GO-AuNPs + AMP, (iv) GO-AuNPs + DNA aptamer + AMP + TMB/H₂O₂, (v) GO-AuNPs + DNA aptamer + MAMP + TMB/H₂O₂, (vi) GO-AuNPs + DNA aptamer + AMP + hemin + TMB/H₂O₂ and (vii) GO-AuNPs + DNA aptamer + MAMP + hemin + TMB/H₂O₂. (C) Enhanced catalytic signal of the GO-CTAB-AuNP hybrid to MAMP detection in comparison to CTAB-AuNPs and GO. [ATS] = 100 μ M. Reaction carried out at room temperature.

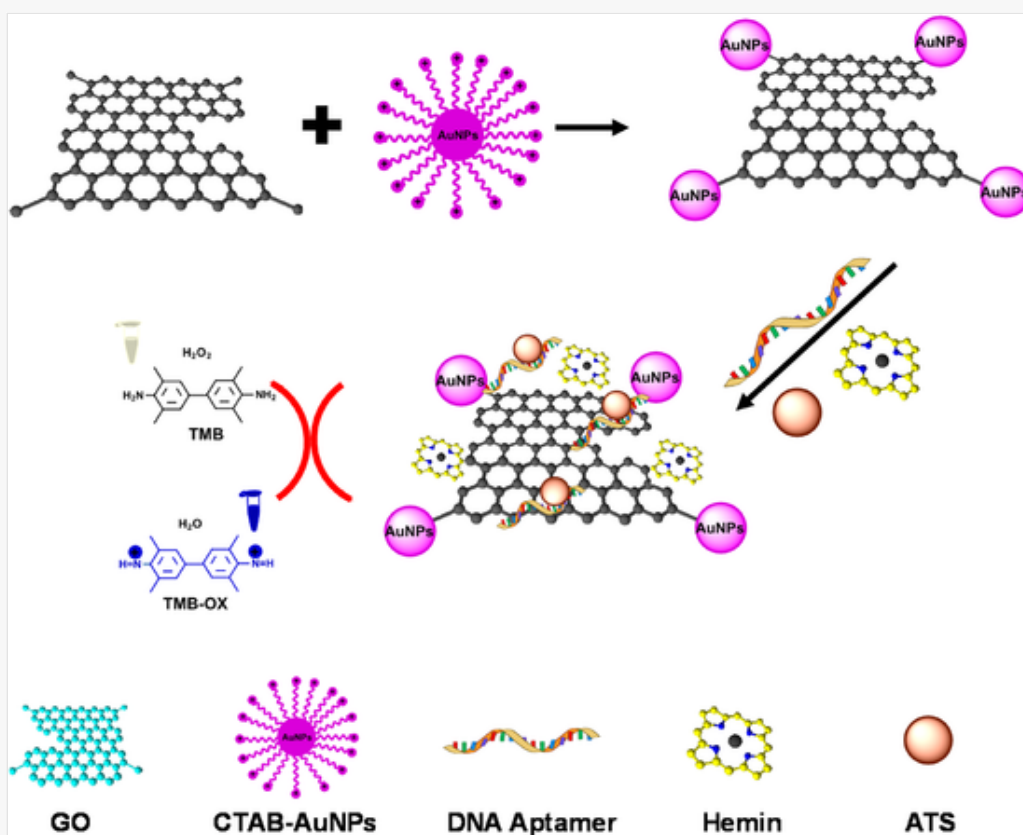
To confirm the catalytic-enhancing property of the hybrid nanozyme, the catalytic signal of GO, CTAB-AuNPs and the GO-CTAB-AuNP hybrid nanozyme to MAMP detection was investigated under the same experimental condition. Fig. 3C shows that the GO-CTAB-AuNPs hybrid nanozyme induced higher catalytic signal for the detection of MAMP in comparison to the signal obtained for GO and CTAB-AuNPs. From the data, it is tentative that the combined catalytic activity of GO and CTAB-AuNPs, embedded within the hybrid nanozyme system, induced the enhanced catalytic signal. Thus, the sensitivity of the biosensor to MAMP detection was enhanced when GO-CTAB-AuNPs hybrid was used as the nanozyme catalyst.

The working principle of the aptamer-based GO-CTAB-AuNP-hemin peroxidase mimic catalytic biosensor for ATS detection is presented in Scheme 1. In general, peroxide-like activity of nanozymes using DNA aptamers as affinity receptors have been reported by several groups [46–49]. The mechanism of peroxidase-like catalytic activity of nanozymes have generally been attributed to the decomposition of H₂O₂ to hydroxyl radicals by the nanozyme catalyst [25]. Firstly, negatively charged GO is bound to the positively charged CTAB-AuNPs via electrostatic interaction. Thereafter, a thiolated DNA aptamer specific to the target ATS was adsorbed onto the GO-CTAB-AuNP surface via either DNA base stacking with GO hydrophobic domains, electrostatic repulsion with GO oxygen-rich domains or hydrogen bonding. Studies have also shown that the pyrimidine

bases (C and T) bind more strongly to GO than the purine bases (G and A), suggesting the possibility that aromatic-based stacking interactions can occur with the bonded GO [50]. To enhance the binding interaction, the DNA aptamer was thiolated to provide the possibility of additional adsorption binding to the CTAB-AuNP surface. The target ATS drug was then added to the biosensor system and captured by the DNA aptamer followed swiftly by the addition of hemin. Hemin binds strongly to graphene via π - π interaction and was used to enhance the catalytic signal of the biosensor. TMB was then added into the aptamer-GO-CTAB-AuNP-hemin system and thereafter catalyzed the oxidization of the hybrid nanozyme in the presence of H_2O_2 , leading to the generation of colored blue product that corresponds selectively to the concentration of the detected ATS drug.

alt-text: Scheme 1

Scheme 1



Schematic representation of the hybrid nanozyme peroxidase mimic aptamer-based biosensor for ATS detection. GO is first bound to CTAB-AuNP via electrostatic interaction to form a GO-AuNP hybrid nanozyme complex. Thereafter, the DNA aptamer is bound to the GO-AuNP hybrid nanozyme and captures the target ATS. Hemin is added to the system to enhance the catalytic signal followed by the catalytic oxidation of TMB by H_2O_2 , generating a blue colour complex specific to the target ATS concentration.

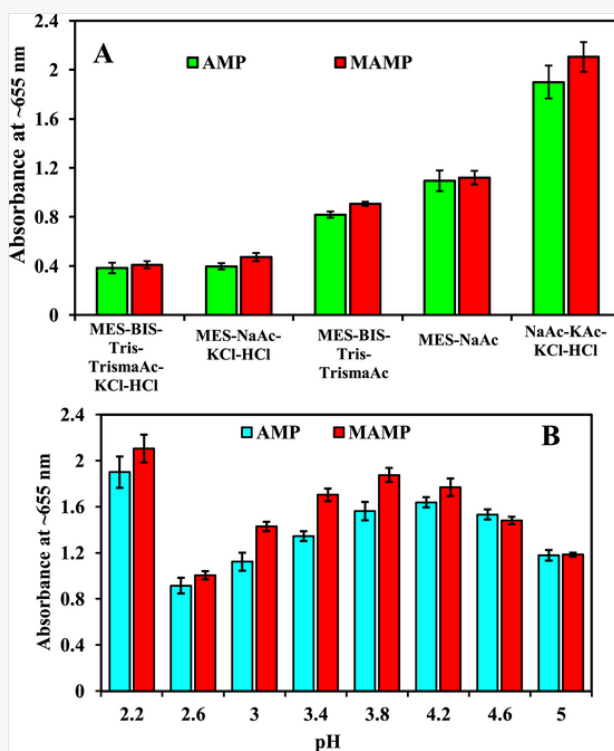
3.4 Optimization

3.4.1 Buffer and pH effect

pH stabilization during enzymatic assays can be accomplished with a buffer solution while the buffer components can influence optimum catalytic efficiency. The term “Good buffers” was coined to represent certain buffers which demonstrated a catalytic stabilizing effect on the enzymatic assay [51]. Finding the appropriate buffer for optimum catalytic efficiency is quite challenging due to the varying degree of analyte interactions for each enzyme assay. To find the appropriate buffer for the targeted ATS detection, we probed the catalytic sensitivity of the aptamer-based GO-CTAB-AuNP-hemin hybrid biosensor in the presence of five different buffer solutions (Fig. 4A). From our investigation, we found that NaAc-KAc-KCl-HCl, pH 2.2 buffer, a novel buffer developed in this work, induced an optimum catalytic signal for AMP and MAMP detection. Hence, this buffer was chosen as the buffer of choice for the ATS catalytic peroxidase mimic assay.

alt-text: Fig. 4

Fig. 4



Catalytic response of the aptamer-based GO-CTAB-AuNP-hemin hybrid nanozyme to AMP and MAMP detection in different buffer solutions (A) and at different pH conditions (buffer: NaAc-KAc-KCl-HCl, pH 2.2) (B). GO-CTAB-AuNP hybrid nanozyme (0.03 nM); 100 μ M DNA aptamer; 100 μ M ATS; 100 μ M hemin; 3000 μ M TMB; 1.2 M H_2O_2 .

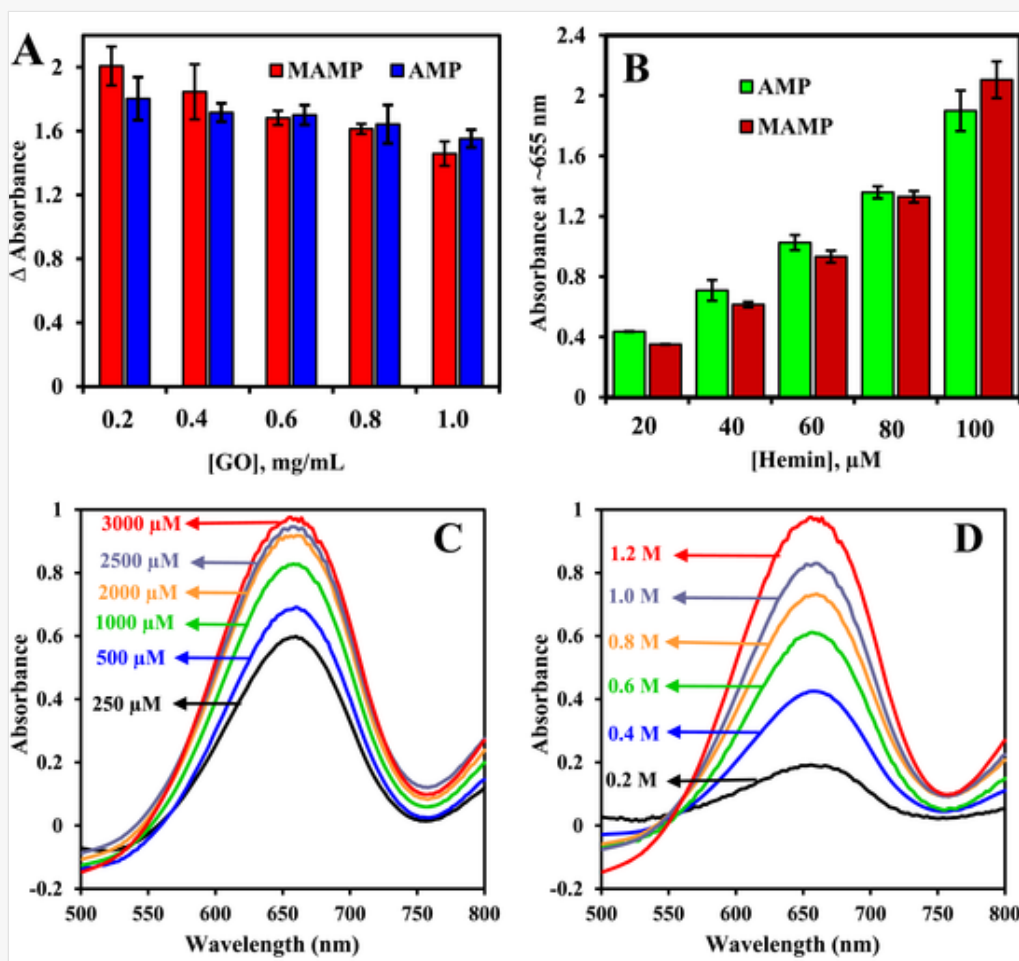
Subsequently, the effect of pH on the catalytic response of the peroxidase mimic biosensor to AMP and MAMP detection was studied. Fig. 4B shows the catalytic response of the aptamer-based GO-CTAB-AuNP-hemin hybrid biosensor to AMP and MAMP in the pH range 2.2–5.0. From the data, optimum catalytic response was observed at pH 2.2, this then decreased at pH 2.6 and increased steadily to pH 3.8 for MAMP and pH 4.0 for AMP and finally, the catalytic response decreased steadily up until pH 5.0. Based on the observed data, pH 2.2 was chosen as the optimum pH condition for the catalytic assay.

3.4.2 Effects of GO and CTAB-AuNPs concentration

The effect of GO concentration on the catalytic efficiency of the aptamer-based GO-CTAB-AuNP-hemin peroxidase mimic biosensor for AMP and MAMP detection was studied. Fig. 5A shows the catalytic response obtained for AMP and MAMP detection at different GO concentration. From the data, we observe a slight decrease in catalytic signal relative to increasing concentration of GO. The absorption spectra showing the effect of GO on the SPR absorption peak of CTAB-AuNPs was also studied. As shown in Fig. S1, we found the intensity of the SPR absorption peak decreased as the concentration of GO increased. Based on these results, we chose 0.2 mg/mL GO as the choice concentration for the peroxidase mimic assay.

alt-text: Fig. 5

Fig. 5



Catalytic response of the aptamer-based GO-AuNP-hemin peroxidase mimic biosensor to MAMP and AMP detection at different (A) GO and (B) hemin concentrations. UV/vis absorption spectra showing the catalytic response of the peroxidase mimic biosensor to MAMP detection at different (C) TMB concentration (H_2O_2 concentration fixed) and (D) H_2O_2 concentration (TMB concentration fixed). GO-CTAB-AuNP hybrid nanozyme (0.03 nM); 100 μ M DNA aptamer; 100 μ M ATS; 100 μ M hemin; 3000 μ M TMB; 1.2 M H_2O_2 .

The effects of CTAB-AuNPs on the catalytic efficiency of the peroxidase mimic assay was also studied using AMP as the target drug. From Fig. S2A, we observe that the catalytic signal of the peroxidase mimic biosensor increased as the concentration of CTAB-AuNPs increased. The absorption spectra (Fig. S2B) also showed that the SPR absorption peak was influenced by the plasmonic NP concentration. From the observed data, we chose 0.04 nM CTAB-AuNPs as the choice concentration for the peroxidase mimic assay.

3.4.3 Effects of hemin concentration

Hemin was used as a catalytic signal enhancer for the peroxidase mimic biosensor. Hence, the effects of hemin concentration on the aptamer-based GO-CTAB-AuNP-hemin peroxidase mimic biosensor for AMP and MAMP detection was studied. A steady increase in catalytic signal was observed with increasing hemin concentration for AMP and MAMP detection as shown in Fig. 5B. Hence, 100 μM hemin was chosen as the optimum concentration for enhanced catalytic reaction.

3.4.4 Effects of TMB and H_2O_2 concentration

The effects of TMB and H_2O_2 concentration on the aptamer-based GO-CTAB-AuNP-hemin peroxidase mimic biosensor was studied. The catalytic signal to MAMP detection was probed in the presence of TMB concentration from 250 to 3000 μM and H_2O_2 concentration from 0.2 to 1.2 M. As shown in Fig. 5C and D, the catalytic absorption peak at around 660 nm, increased steadily as the concentration of TMB and H_2O_2 increased in the biosensor system. We observed a higher margin of difference in catalytic signal for increasing concentration of H_2O_2 than for TMB. This indicates that an increasing concentration of H_2O_2 had a more significant impact on the catalytic signal of the peroxidase mimic biosensor than an increasing concentration of TMB. Based on the observed results, 3000 μM TMB and 1.2 M H_2O_2 were chosen as the optimum substrate and oxidant concentrations for the peroxidase mimic biosensor.

3.5 Nanozyme kinetic bioassay

Kinetic studies of the aptamer-based GO-CTAB-AuNP-hemin peroxidase mimic biosensor was carried out to reveal changes in the velocity of the reaction as a function of changes in the substrate concentration at a fixed enzyme concentration [52]. Firstly, the absorbance vs time plots for varying TMB substrate concentration (fixed H_2O_2 concentration) and H_2O_2 concentration (fixed TMB concentration), were used to determine the initial rate of reaction (v) (i.e., the slope of the graph), the value of v was then divided by the extinction coefficient of TMB ($3.9 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) [53]. The Michaelis-Menten nonlinear curve was obtained according to equation (1) by plotting v vs varying concentration of TMB (fixed H_2O_2 concentration) (Fig. S3A) and H_2O_2 (fixed TMB concentration) (Fig. S3C):

$$v = \frac{V_{max} \cdot [S]}{[S] + K_m}$$

(1)

The linear curve was fitted by plotting $\frac{1}{v} = \frac{K_m}{V_{max} \cdot [S]} + \frac{1}{V_{max}}$ versus $\frac{1}{[S]}$ (Figs. S3B and D) according to the Lineweaver-Burke equation [52]:

$$\frac{1}{v} = \frac{K_m}{V_{max} \cdot [S]} + \frac{1}{V_{max}} \quad (2)$$

V_{max} is the maximum reaction velocity, K_m is the Michaelis-Menten constant and $[S]$ is the substrate concentration. The intercept of the plot is equal to $\frac{1}{V_{max}}$ and was used to determine the value of V_{max} while K_m was determined by multiplying the slope value with V_{max} .

Table 1 shows the V_{max} , K_m and K_{cat} values obtained for MAMP and AMP detection in the presence of TMB and H_2O_2 . K_m is the measure of catalytic affinity between the hybrid nanozyme and substrate in which a low K_m value represents a strong affinity and vice versa; V_{max} is a measure of the catalytic rate of reaction in which a higher V_{max} value represents a higher catalytic rate of reaction; K_{cat} is the catalytic constant whose value relates to the maximum number of colored products (oxidized substrate) “turned over” per nanozyme per second ($K_{cat} = V_{max}/[E]$, where E is the hybrid nanozyme concentration. From the kinetic assay results (Table 1), we observed a stronger affinity between the hybrid nanozyme and the substrate (TMB) for AMP detection based on the lower K_m value (0.24 mM) obtained compared to MAMP ($K_m = 0.52$ mM). However, the higher K_{cat} value obtained for MAMP detection ($K_{cat} = 8.2 \times 10^5 \text{ s}^{-1}$) in the presence of TMB substrate, suggests there was higher amount of oxidized substrate generated per nanozyme per second relative to AMP ($K_{cat} = 2.5 \times 10^5 \text{ s}^{-1}$). Generally, we observed a higher K_{cat} value for MAMP detection relative to AMP in the presence of both TMB and H_2O_2 . Our work is the first to report on the K_m , V_{max} and K_{cat} values for enzymatic catalytic reaction of MAMP and AMP using the novel GO-CTAB-AuNP-hemin hybrid nanozyme.

alt-text: Table 1

Table 1

i The table layout displayed in this section is not how it will appear in the final version. The representation below is solely purposed for providing corrections to the table. To preview the actual presentation of the table, please view the Proof.

K_m , V_{max} and K_{cat} kinetic parameters of GO-CTAB-AuNP hybrid nanozyme for MAMP and AMP detection.

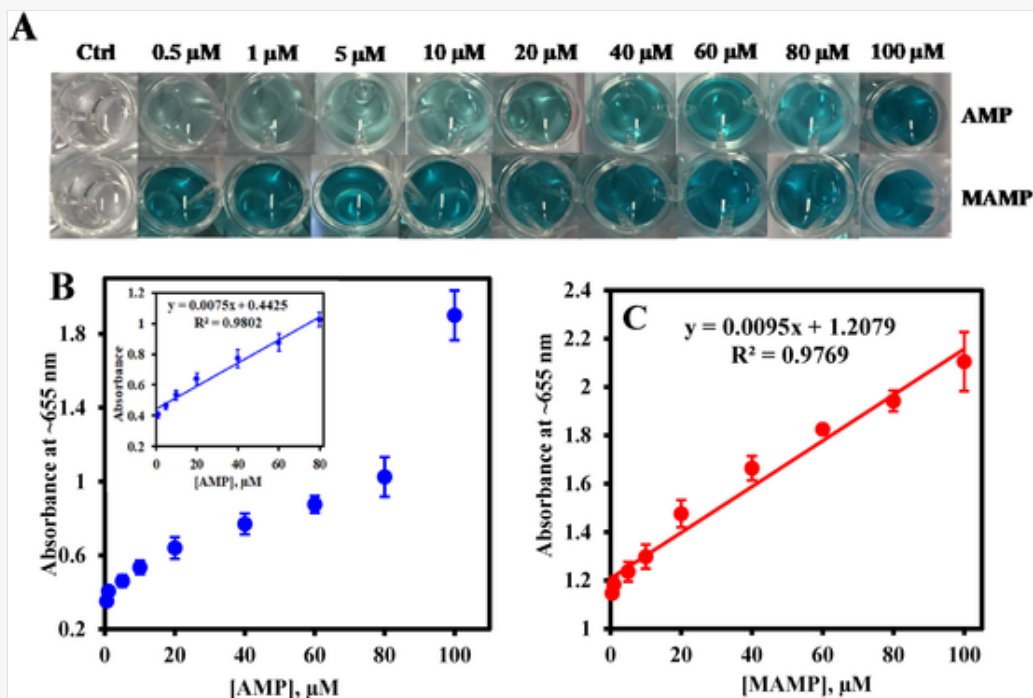
Drug	[E] M	Substrate	V_{max} (M s ⁻¹)	K_m (mM)	K_{cat} (s ⁻¹)
MAMP	2.7×10^{-11}	TMB	2.2×10^{-5}	0.52	8.2×10^5
MAMP	2.7×10^{-11}	H_2O_2	3.1×10^{-5}	1100	1.2×10^6
AMP	2.7×10^{-11}	TMB	6.8×10^{-6}	0.24	2.5×10^5
AMP	2.7×10^{-11}	H_2O_2	8.5×10^{-6}	396	3.2×10^5

3.6 Quantitative detection of AMP and MAMP

Quantitative detection of AMP and MAMP using the aptamer-based GO-CTAB-AuNP-hemin peroxidase mimic biosensor was carried out in the concentration range of 0.5–100 μM . Fig. 6A shows the quantitative colorimetric response of 0.5–100 μM AMP and MAMP detection using the peroxidase mimic biosensor. From the colour reaction, we observed a clear change in the colour intensity as the detected concentration of AMP increased. Whereas for MAMP, the intensity of the colour reaction was less prominent but clearly distinguishable in the catalytic signal. The difference may be attributed to the very strong oxidation of TMB in the presence of MAMP. From Table 1, we observed that the K_{cat} value for MAMP in the presence of the substrate TMB and H_2O_2 was higher than the value obtained for AMP. This proves to show that the aptamer-based GO-CTAB-AuNP-hemin peroxidase mimic biosensor exhibits higher catalytic activity to MAMP than AMP.

alt-text: Fig. 6

Fig. 6




Quantitative detection of the target AMP and MAMP. (A) Photographic colorimetric response and catalytic calibration plot for quantitative detection of AMP (B) and MAMP (C). Inset of Fig. 7B is the linear calibration curve for AMP detection. GO-CTAB-AuNP hybrid nanozyme (0.03 nM); 100 μM DNA aptamer; 100 μM ATS; 100 μM hemin; 3000 μM TMB; 1.2 M H_2O_2 . Data recorded at ~1 min.

The corresponding catalytic signal curve shown in Fig. 6B for AMP and Fig. 6C for MAMP revealed quantitative detection of the drug sample. As the concentration of the target ATS increased in the detection system, the catalytic signal progressively increased. From the linear calibration curve, we determined the limit

of detection (LOD) by multiplying the standard deviation of blank measurement by 3 and dividing by the slope of the linear calibration graph. The LOD obtained for AMP detection was 185 nM (34.1 ng/mL) and 154 nM (28.6 ng/mL) for MAMP detection. Table 2 shows the improved sensitivity of the peroxidase mimic biosensor over most published colorimetric data for AMP and MAMP detection. From the comparison (Table 2), the response time (~1 min) of our peroxidase mimic biosensor is the best reported to date.

alt-text: Table 2

Table 2

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Comparison of the analytical performance of the aptamer-GO-CTAB-AuNP-hemin hybrid nanozyme biosensor probe for ATS detection with published data.

Probe	Drug	Method	LOD	Detection time	Ref.
Solid PDMS-TEOS-SiO ₂ matrix	AMP	Colorimetry	5×10^6 ng/mL	10 min	[4]
	MAMP	Colorimetry	4×10^6 ng/mL	10 min	
Au@Ag DNA aptamer probe	MAMP	Colorimetry	0.0149 ng/mL	15 min	[56]
Simons reagent in sol-gel matrix	MAMP	Colorimetry	2.1×10^5 – 5.9×10^5 ng/mL	2 min	[57]
Centrifugal microdevice	MAMP	Colorimetry	7.5×10^5 ng/mL	5 min	[12]
G-quadruplex-hemin DNAzyme molecular beacon	MAMP	Colorimetry	0.0746 ng/mL	10 min	[58]
Aptamer-GO-CTAB-AuNP- hemin hybrid nanozyme	AMP	Colorimetry	34.1 ng/mL	1 min	This work
	MAMP	Colorimetry	28.6 ng/mL	1 min	

3.7 Detection and quantitation of AMP in a seized sample

The efficacy of the aptamer-based GO-CTAB-AuNP-hemin peroxidase mimic biosensor was probed for the detection and quantitation of AMP in a seized drug sample. Fig. S4A shows the colorimetric response of the peroxidase mimic biosensor to different AMP concentrations within the sample. It is evident that the colour intensity increased steadily as the concentration of AMP increased. The corresponding quantitative catalytic signal curve presented in Fig. S4B revealed the steady increase in catalytic signal as the concentration of AMP increased. From the catalytic signal plot, we determined the LOD to be 187 nM (34.5 ng/mL) which was close to the LOD obtained for standard AMP sample (185 nM (34.1 ng/mL)).

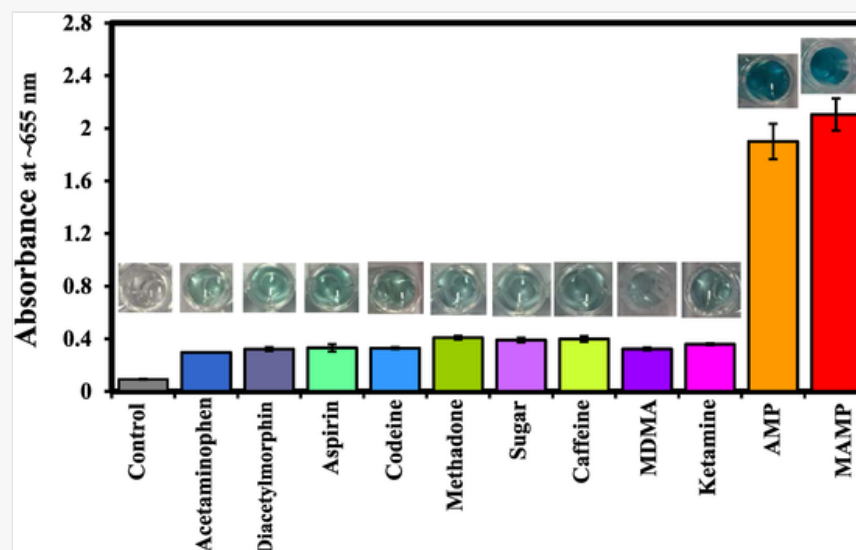
3.8 Selectivity of the peoxidase-mimic bioassay

Marquis, Simon's and Mandelin reagents are the standard presumptive tests used in ATS analysis. Marquis and Mandelin reagents are specifically used for AMP and MAMP while Simon's reagent is used to differentiate between MAMP and AMP [54]. However, there are also several other substances that respond positively to both the Marquis and Mandelin reagents [55] and as such a more selective presumptive test designed specifically for AMP and MAMP would be very advantageous. To probe the selectivity of the peroxidase mimic biosensor to AMP and MAMP, known substances and drugs that respond positively to the Marquis and Mandelin reagents were tested. Table S1 provides information on the positive response of the tested drugs and substances to Marquis and Mandelin reagents and the colour they exhibit.

Fig. 7 shows the colorimetric response and catalytic signal obtained for AMP and MAMP in comparison to the data obtained for the tested substances and drugs. From the data, we observed a very strong catalytic signal for AMP and MAMP accompanied with a very deep blue colorimetric reaction. In contrast, a very weak catalytic response was exhibited by the additional tested substances and drugs which could be differentiated from the deep blue colour exhibited by AMP and MAMP. In terms of the catalytic signal efficiency, the AMP catalytic signal was 4-fold higher while MAMP was at most 5-fold higher than the catalytic signal obtained for the tested interferents. Based on our results, it is reasonable to conclude that the presumptive testing of AMP and MAMP can be selectively differentiated from other tested interferents by the rapid deep blue colour reaction transduced by the aptamer-based GO-CTAB-AuNPs peroxidase mimic biosensor.

alt-text: Fig. 7

Fig. 7




Catalytic and corresponding colorimetric response of the aptamer-GO-AuNP-hemin hybrid nanozyme to AMP and MAMP in comparison to other tested drugs and substances. Concentration of ATS and other non-targets = 100 μM ; GO-CTAB-AuNP hybrid nanozyme (0.03 nM); 100 μM DNA aptamer; 100 μM hemin; 3000 μM TMB; 1.2 M H_2O_2 . Data recorded at ~1 min.

3.9 Detection in mixed samples

The efficacy of the aptamer-based GO-CTAB-AuNP-hemin peroxidase mimic biosensor to detect the targeted ATS in mixed drug samples was investigated. Known adulterants such as acetaminophen, caffeine and ketamine were mixed with different concentration of MAMP and the analytical performance of the aptamer-based nanohybrid peroxidase mimic biosensor was examined. Table 3 shows the recovery efficiency of the aptamer-based nanohybrid peroxidase mimic biosensor for the detection of 100, 60 and 20 μM MAMP in a mixed sample solution containing a fixed concentration of added adulterant (100 μM). From the data, the recovery efficiency was at most 79.3% for 100 μM of added MAMP in acetaminophen, 78.8% for 20 μM of added MAMP in caffeine and 79.6% for 60 μM of added MAMP in ketamine. In general, we found out that the aptamer-based GO-CTAB-AuNP-hemin peroxidase mimic biosensor could detect MAMP in mixed drug samples with varying efficiency.

alt-text: Table 3

Table 3

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Analytical performance of the aptamer-GO-CTAB-AuNP-hemin hybrid nanozyme peroxidase-like catalytic biosensor for MAMP detection in mixed drug samples. All data recorded at ~ 1 min.

Mixed drug (100 μM)	MAMP added (μM)	Found (μM)	Recovery (%) \pm SD (%)	RSD (%)
Acetaminophen	100	79.3	79.3 ± 4.7	4.7
	60	36.5	60.8 ± 1.8	3.0
	20	13.7	68.6 ± 0.9	1.7
Caffeine	100	70.9	70.9 ± 5.7	6.4
	60	37.8	63.1 ± 0.4	0.6
	20	15.8	78.8 ± 1.4	2.3
Ketamine	100	71.2	71.2 ± 1.9	2.0
	60	47.8	79.6 ± 0.9	1.2
	20	15.5	77.6 ± 0.3	0.4

SD = Standard deviation of three replicate measurements.

4 Conclusions

Negatively charged GO was electrostatically bonded to positively charged CTAB-AuNPs and the formed GO-CTAB-AuNP hybrid nanozyme was employed in an aptamer-based peroxidase mimic biosensor assay for the

enhanced catalytic colorimetric detection of AMP and MAMP. Hemin, an iron-containing porphyrin complex was incorporated into the biosensor system and used as a catalytic signal amplifier. The structural and optical properties of GO, CTAB-AuNPs and the GO-CTAB-AuNP hybrid nanozyme were characterized using electron-based and optical techniques. Under the optimum reaction conditions, AMP and MAMP were quantitatively detected with high sensitivity and the selectivity of the biosensor was confirmed by the deep blue colour reaction specific to AMP and MAMP recognition.

CRediT authorship contribution statement

Oluwasesan Adegoke: Conceptualization, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Svetlana Zolotovskaya:** Formal analysis, Writing - review & editing. **Amin Abdolvand:** Formal analysis, Writing - review & editing. **Niamh Nic Daeid:** Conceptualization, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.talanta.2020.120990>.

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The corrections made in this section will be reviewed and approved by a journal production editor. The newly added/removed references and its citations will be reordered and rearranged by the production team.

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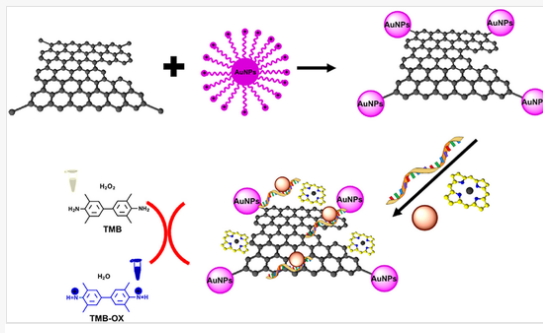
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Graphical abstract

alt-text: Image 1



Highlights

- GO-multi-shaped cationic AuNP-hemin hybrid nanozyme was newly developed.
- An anti-ATS DNA aptamer was bonded to the hybrid nanozyme.
- Hemin was used as a catalytic-induced signal amplifier for the colorimetric assay.
- TMB catalyzed reaction of H₂O₂ by the aptamer-based hybrid nanozyme detected AMP and MAMP.
- The target drugs were detected within one minute with excellent selectivity and ultrasensitivity.

Appendix A Supplementary data

The following is the Supplementary data to this article:

[Multimedia Component 1](#)

Multimedia component 1

alt-text: Multimedia component 1

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